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Physiology and Molecular Phylogeny of Bacteria Isolated from Alkaline Distillery Lime

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Abstract

This paper presents the results of the research on the number, taxonomic composition, and biochemical properties of bacterial strains isolated from the alkaline Solvay distillery lime, deposited at the repository in Janikowo (central Poland). Fifteen strains out of 17 were facultative alkaliphiles and moderate halophiles, and two were alkalitolerants and moderate halophiles. The number of aerobic bacteria cultured in alkaline lime was approximately 10^5 CFU ml^{-1}, and the total number of bacteria was 10^7 cells g^{-1}. According to 16S rRNA gene sequence analysis, nine strains belonged to the genus *Bacillus,* six to the genus *Halomonas,* one to the genus *Planococcus*, and one to the genus *Microcella*. Strains that hydrolyse starch and protein were the most numerous. Esterase (C4) and esterase lipase (C8) were detected in the majority of bacterial strains. Twelve strains exhibited α-glucosidase activity and nine, naphtol-AS-BI-phosphohydrolase activity. The present study proves that alkaliphilic bacteria of this type may constitute a source of potentially useful extremozymes.

K e y w o r d s: *Bacillus* sp., *Halomonas* sp., alkaline solvay distillery lime, alkaliphiles, halophiles

Introduction

Extreme environments constitute a habitat for microorganisms with unique biochemical properties, useful for the industries, medicine, and environmental protection. In recent years the knowledge of extremophilic microorganisms which populate ecosystems generally considered unusual, has improved dramatically (Quazi, 2013). The main reason for the extensive research on extremophiles is the biotechnological potential of their cells and metabolites. These microorganisms are source of several biotechnologically relevant enzymes and biomolecules, *e.g.* cellulases, amylases, and ectoine (Jones *et al*., 1998; Trotsenko and Khmelenina, 2002; Grant and Heaphy, 2010).

Microorganisms populating highly alkaline environments constitute a diversified group and can be divided into alkaliphiles and alkalitolerants. Alkaliphiles are capable of living in the environment with a pH of 10 and higher, thriving, however, in the environment with a pH of around 9 (their optimum pH), but incapable of surviving in the environment with a pH of 7 or lower. On the other hand, alkalitolerants are capable of living in the environment with a pH higher than 10, although they thrive in environments with pH of 7 (their optimum pH is neutral) (Krulwich, 1986).

Alkaliphiles are found in natural alkaline environments such as saline soda lakes and ponds of East Africa (Foti *et al*., 2006), Europe (Gerasimenko *et al*., 2003), Asia (Antony *et al*., 2013), Ca^{2+} alkaline springs of Near East, and soda deserts of North America (Duckworth *et al*., 1996). Marine environments contain many alkalitolerant bacteria and usually a smaller number of alkaliphiles (Ntougias *et al*., 2006; Tamura *et al*., 2013). Alkaliphiles have also been isolated from a number of alkaline wastes, by-products from food processing, *e.g.* potato processing plant effluents (Collins *et al*., 1983), olive and maize processing wastewater (Ntougias *et al*., 2006; Sanchez-Gonzalez *et al*., 2011).

Although alkaliphiles have been thoroughly studied, no information has been found about their isolation from a lime sludge, a by-product in Solvay soda process.

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Alkaline distillery lime deposited in sludge ponds on the premises of the manufacturing plant in Inowrocław and Janikowo (central Poland) is a potential habitat of alkaliphilic and alkalihalotolerant microorganisms due to its highly alkaline character (pH from 9.0 to 11.4) and increased salt concentration (up to 9% in dry matter). The main component of the alkaline lime is calcium carbonate (above 90%) and the remaining include amorphous calcium hydroxide (6%), silicon dioxide, magnesium, aluminum and chlorides (Ziółkowska *et al.*, 2013). Owing to strong alkaline properties, distillery lime increases the pH of aqueous solutions up to 11–12 (Jeliński *et al.*, 2011).

The aim of this research was to determine the abundance, physiology and molecular phylogeny of extremophilic microbiota originating from alkaline distillery lime.

Experimental

Materials and Methods

Study area. Alkaline distillery lime has been deposited in sludge ponds in Janikowo (central Poland, Kuyavia) since 1957. The repository of alkaline distillery lime, composed of several ponds separated by causeways, covers an area of 200 ha and has the capacity of over 13,000,000 Mg. It rises up to 16 m above the ground level (Regional Inspectorate for Environmental Protection, 2013).

Sampling sites. Alkaline distillery lime was collected in September 2012 from three ponds of different salinity: sampling site S (slightly saline), M (moderately saline), H (highly saline). Samples were collected using a Total Groundcare soil sampler (Zarzecze, Poland) from the surface layer of the deposits (depth of 0–20 cm) from plots with an area of 25 m^2. Placed in sterile glass jars, they were transported to the laboratory in a portable ice bag at the temperature below 4°C. In the laboratory, the samples from a particular sampling site were mixed thoroughly and subsamples for subsequent microbial and physico-chemical tests were placed in sterile glass jars. The analyses started within 4 hours of sample collection.

Chemical analysis of distillery lime. Gravimetric moisture of alkaline lime was determined with the method of mass loss in drying (Radojevic and Bashkin, 2006). Before the analysis, solid and dried samples were hammered to reduce agglomeration, then disintegrated and homogenized in a roller mill. The pH values of the samples were measured in an aqueous suspension using a deposit: solution ratio of 1 : 1 (w/w). Elmetron CPC-501 pH meter (Zabrze, Poland) was used for pH determination in the aqueous phase. The concentration of chloride ions was determined by argentometric titration with silver nitrate (Radojevic and Bashkin, 2006). Total organic carbon (TOC) was measured in TOC 5000 analyzer combined with the SSM-5000A module (Shimadzu, Kyoto, Japan). TOC was calculated by subtracting inorganic carbon concentration (IC) from total carbon concentration (TC). Organic nitrogen concentration was calculated by subtracting ammonia nitrogen from total Kjeldahl nitrogen (TKN). TKN was determined in a Turbotherm system (Gerhardt, Konigswinter, Germany). Ammonia nitrogen concentration was measured using Nessler method (Spectroquant Merck SQ 118, Darmstadt, Germany), after distillation in Gerhardt Vapodest 20 system (Gerhardt, Konigswinter, Germany). Nitrate concentration was determined using spectrophotometric method (Spectroquant Merck SQ 118, Darmstadt, Germany) after extracting nitrate from the sample using 2.0 M KCl. Total phosphorus content was measured with the spectrophotometric molybdenum blue method (Spectroquant Merck SQ 118, Darmstadt, Germany) after sample digestion with aqua regia.

Isolation and enumeration of bacteria. To isolate bacterial populations from alkaline lime, 10 g from the three mixed samples was added into 90 ml of NaCl solution (concentration corresponding to the chloride content in a given sample). The mixtures were blended thoroughly, tenfold serial dilutions were prepared and 0.1 ml of each suspension was spread onto three replicate plates with the appropriate culture media.

Extracts from the alkaline lime (100 g dissolved in 1 l of distilled water, mixed for 20 min, filtered and adjusted to pH 7 and 11 using the appropriate buffer solutions according to Ntougias *et al.* (2006), were solidified with agar and used to estimate the number of cultivable bacteria. All plates were incubated up to 7 days at 25°C. The number of grown colonies was expressed as colony forming units (CFU) per gram (dry weight) of alkaline lime. To prepare bacterial strain collection intended for further physiological and taxonomy studies, single colonies appearing on isolation plates were subcultured in a medium containing (g l^{-1}) glucose 5, yeast extract 1, $MgSO_4$ 0.01, and NaCl 30, supplied with appropriate buffer system. The isolation was based on the following characteristics of bacterial colonies: color, size, shape, and prevalence on the medium.

The total number of bacteria in alkaline lime was determined by acridine orange direct count (AODC) technique after Hobbie *et al.* (1977). For this purpose, 1 g of alkaline lime (fixed with formaldehyde – 3.8% v/v final concentration, sterilized with MF-Millipore Membrane Filters, 0.22 μm) was placed in glass tubes containing 9 ml of filter-sterilized citric acid solution (final concentration 0.6 M), added to dissolve calcium carbonate in the samples. The suspensions were vortexed for 2 min. Triplicate subsamples were stained with

acridine orange (final concentration 0.01%) for 3 min (Hobbie *et al.*, 1977). At least 100 cells or all cells in 100 fields of view were counted under a microscope (Nicon Eclipse E200, Japan) for each slide.

Statistical analysis was conducted using Statistica 6.0. software and analysis of variance (ANOVA). The impact of two independent factors (pH and chloride concentration) on the number of cultivable bacteria and the total number of bacteria in alkaline distillery lime was compared. ANOVA was followed by Tukey's test (HSD).

Physiological studies. Cell morphology and the purity of cultures were checked routinely using a Nikon light microscope (Japan). All experiments were performed at 25°C, unless stated otherwise. Alkaliphilic/alkalitolerant strains were maintained and grown routinely on alkaline media (Ntougias *et al.*, 2006), composed of the nutritional base described above, 30 g l^{-1} NaCl, 0.1 mM $MgSO_4$ and a buffer (Na_2CO_3-K_2HPO_4), at pH 10.

Bacterial tolerance to salt was investigated in media containing the nutritional base and 0.1 mM $MgSO_4$ with different NaCl concentrations (0, 30, 50, 80, 100, 150, 200 and 250 g l^{-1} NaCl). Bacterial growth was measured as optical density at 600 nm after 7 days.

Nutrient media containing specific substrates (sodium caseinate, olive oil, starch, carboxymethyl cellulose), all at a concentration of 0.5% (w/v), 0.1 mM $MgSO_4$ and 0.05 g l^{-1} yeast extract were used to evaluate the organic substrate utilization by the isolates. Frazier reagent ($HgCl_2$ 12 g, distilled water, 80 ml, concentrated HCl, 20 ml) was used to detect protein hydrolysis, Rhodamine B (1 mg ml^{-1}, Sigma, added to the medium after sterilization, followed by incubation and UV irradiation) to evaluate fat hydrolysis, Gram iodine to assess starch hydrolysis, and 0.1% Congo red and 1 M NaCl to determine cellulose hydrolysis. Alkaliphilic/alkalitolerant strains were investigated at pH 10 and 3% NaCl. The medium containing NaCl (pH 10) was used to investigate the growth temperature range (15, 20, 30, 40, 50°C). Anaerobic growth was tested using the BioMerieux GENbox anaerobic system at 25°C for 7 days. The ability of individual isolates to synthesize constitutive enzymes was studied using API ZYM system (BioMerieux, France).

Identification of bacterial strains based on the 16S rRNA gene. Total genomic DNA was extracted from the strains using the G-Spin Total DNA Extraction Kit (iNtRON Biotechnology, Sungnam, Korea) following the instructions given by the manufacturer. The 16S rRNA gene was amplified by PCR in a reaction mixture containing 1 U DreamTaq DNA polymerase (Thermo Scientific, Waltham, MA, USA), 0.2 mM dNTP mixture (Thermo Scientific), 1 × DreamTaq buffer (Thermo Scientific), 0.325 µM of primers 27F 5'-AGA GTT TGA TCM TGG CTC AG-3', (Lane, 1991) and 1492R 5'-TAC GGY TAC CTT GTT ACG ACT T-3' (Polz and Cavanaugh, 1998), 10 µg BSA (Thermo Scientific) and 1 µL genomic DNA in a total volume of 25 µL. Thermal profile consisted of an initial denaturation at 98°C for 5 min, 32 cycles of denaturation at 94°C for 0.5 min, annealing at 48°C for 0.5 min and extension at 72°C for 1.5 min, and a final extension at 72°C for 10 min. PCR amplicons were checked under UV excitation on a 1 w/v% agarose gel stained with GR Safe DNA Stain (Innovita, Gaithersburg, MD, USA). PCR product purification, sequencing reaction with primer 27F and capillary electrophoresis were performed by the LGC Genomics GmbH (Berlin, Germany). Manual correction of automatic base calling on chromatograms was carried out using the Chromas software v 1.45 (Technelysium, Brisbane, QLD, Australia). Nucleotide sequences obtained in this study were submitted to GenBank under the accession numbers KJ870238-KJ870254.

Closest related species were identified with the EzTaxon online tool (Kim *et al.*, 2012). Sequence alignment with sequences retrieved from GenBank was performed with the SINA Aligner (Pruesse *et al.*, 2012). Phylogenetic analysis including the search for the best-fit model was conducted with the MEGA 6.0 software (Tamura *et al.*, 2013).

Results

All three types of distillery lime had alkaline pH (ranging from 10.2 to 11.3), measured in a water suspension (Table I). Chloride content ranged from 2.88%

Table I
Chemical properties of alkaline distillery lime

Sample	pH	Chlorides (%)	Water content (%)	Total organic carbon (%)	Organic nitrogen (%)	Nitrates (%)	Total phosphorous (%)
S	10.2 ± 0.3	2.88 ± 0.16	62.3 ± 5.5	0.34 ± 0.11	0.001	< LOD	0.006 ± 0.0001
M	10.5 ± 0.2	4.57 ± 0.33	42.3 ± 3.7	nm	nm	nm	nm
H	11.3 ± 0.4	6.40 ± 0.46	45.2 ± 4.1	0.32 ± 0.09	0.001	< LOD	0.008 ± 0.001

LOD – limit of detection, nm – not measured

Table II
Abundance of bacteria in alkaline distillery lime

Sample	Cultivable bacteria (CFU · 10^5 g dry mass)		Total number of bacteria (cells · 10^7 g dry mass)
	pH = 7	pH = 10	
S	14.22 ± 1.19	2.74 ± 1.07	4.39 ± 1.72
M	4.59 ± 0.36	1.47 ± 0.11	18.29 ± 0.89
H	2.16 ± 0.37	1.06 ± 0.21	2.16 ± 0.93

to 6.40% and the water content ranged from 42.3% to 62.3%. The samples contained low amount of nutrients because total organic carbon content was low (only 0.34%). The organic nitrogen and total phosphorous contents were 0.001% and ~0.007%, respectively. The nitrate concentration was below the detection limit.

Two-way analysis of variance showed that both the number of cultivable microorganisms and the total number of bacteria in alkaline lime depended on its pH and chloride concentration ($p < 0.05$).

The results indicate that bacterial number at pH 7 ranged from 2.16 to ± 0.37 CFU · 10^5 g^{-1}_{dw} in highly saline samples and from 14.22 to ± 1.16 CFU · 10^5 g^{-1}_{dw} in slightly saline samples (Table II); at pH 11 these values ranged from 1.06 ± 0.21 CFU · 10^5 g^{-1}_{dw} in highly saline samples and from 2.74 to ± 1.07 CFU · 10^5 g^{-1}_{dw} in slightly saline samples.

Nine strains were isolated from the highly saline samples (marked H, strains H5-H13), four from the moderately saline samples (marked M, strains M1-M4), and four from the slightly saline samples (marked S, strains S14-S17).

The total number of bacteria determined by direct cell count ranged from 2.16 ± 0.93 cells · 10^7 g^{-1}_{dw} in the highly saline samples to 18.29 ± 0.89 cells · 10^7 g^{-1}_{dw} in the moderately saline samples (Table III).

The majority of the bacterial isolates stained Gram-positive. Strains M2, M3, M4, H12, S14 and S15 were Gram-negative, which corresponds to the results of the molecular taxonomic identification. Strains M1, H6-H10 and H13 formed endospores. All strains were strictly aerobic (except strain S16) and oxidase-positive.

Physiological characteristics of bacteria isolated from alkaline distillery lime are presented in Table III. Nine bacterial strains (M1- M3, H5, H7, H8, H10, H11, H13) were able to grow at pH 7–11, and six (M4, H9, H12, S15-S17) at pH 6–11. Efficient growth of most strains was observed at the highest tested pH values and their optimal growth at pH 9–10 (facultative alkaliphiles). Only two strains (H6 and S14) had optimum pH of 7, but showed tolerance of pH values of up to 11 (alkalitolerant bacteria).

Ten isolates tolerated up to 200 g l^{-1} NaCl, seven tolerated concentrations of up to 150 g l^{-1} NaCl. Only one bacterial strain (S16) had low salt tolerance (up to 50 g l^{-1} NaCl). Optimum salt concentration for growth of all the isolates were between 30 and 50 g l^{-1} NaCl therefore they can be regarded as moderate halophiles.

Table III
Physiological characteristics of isolates from alkaline distillery lime

Isolate	pH range	NaCl (%, w/v)	Temperature (°C)	Degradation of organic compounds zone of hydrolysis (mm)			
				Starch	Protein	Lipids	Cellulose
M1	[a]7–11 (9)	0–20 (5)	[a]5–40 (30)	+15	+31	–	–
M2	6–11 (7)	0–20 (5)	5–40 (30)	–	–	–	–
M3	7–11 (9)	3–15 (5)	5–40 (20)	–	–	–	–
M4	6–11 (9)	0–20 (5)	10–40 (30)	–	–		–
H5	7–11 (9)	0–20 (3)	5–50 (30)	+20	+35	+35	–
H6	[a]7–11 (9)	0–15 (3)	5–40 (20)	+12	–	–	–
H7	7–11 (11)	0–20 (5)	5–40[a] (20)	+12	+37	+33	–
H8	[a]7–11 (9)	0–15 (3)	5–40 (30)	+6	+/–	–	–
H9	[a]7–11 (10)	3–15 (3)	5–40 (20)	+18	+51	–	–
H10	[a]7–11 (10)	0–20 (3)	5–40 (20)	–	–	–	–
H11	7–11 (9)	0–20 (5)	5–40[a] (30)	+16	+40	+30	–
H12	7–11 (7)	0–20 (5)	[a]5–40 (20)	+/–	–	–	–
H13	[a]7–11 (9)	0–15 (3)	5–40 (30)	+18	–	–	–
S14	6–11 (7)	0–15 (5)	10–40 (20)	+14	–	–	–
S15	6–11 (9)	0–20 (3)	5–40 (30)	–		–	–
S16	6–11 (10)	0–5 (0)	20–30 (30)	–	–	+4	–
S17	6–11 (9)	0–15 (3)	10–40 (30)	–	+25	–	–

[a] – marginal growth

Table IV
Constitutive enzyme production

Enzyme	Isolate																
	M1	M2	M3	M4	H5	H6	H7	H8	H9	H10	H11	H12	H13	S14	S15	S16	S17
Alkaline phosphatase	–	–	+	+	–	–	–	+	–	+	–	+	–	+	–	+	–
Acid phosphatase	–	–	+	–	–	–	–	+	–	+	+	+	+	–	–	–	–
Esterase (C4)	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+
Esterase Lipase (C8)	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+
Lipase (C14)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
Leucine arylamidase	+	–	+	+	–	–	–	–	+	–	+	+	+	+	–	+	–
Valine arylamidase	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–
Cystine arylamidase	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Trypsin	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
α-chymotripsin	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Naphtol-AS-BI-phosphohydrolase	+	+	+	–	–	+	–	+	+	–	+	–	+	–	–	+	–
α-galactosidase	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–
β-galactosidase	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
β-glucuronidase	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
α-glucosidase	+	–	–	+	+	+	–	–	+	+	+	+	+	+	–	+	+
β-glucosidase	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
N-acetyl-β-glucosaminidase	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
α-mannosidase	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
α-fucosidase	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

The optimum temperature for growth of th e studied isolates (mesophiles) ranged from 20 to 30°C. However, most strains (except M4, S14, S16 and S17) could grow at 5°C.

Strains capable of hydrolyzing starch were the most numerous. All strains isolated from highly saline lime (H5-H13, except H10), plus strains M1 and S14 hydrolyzed this polysaccharide. Seven bacterial strains (M1, H5, H7, H9, H11, S17, H18) hydrolyzed protein, four (H5, H7, H11, S16) hydrolyzed fat while none hydrolyzed cellulose.

The results of the APIZYM tests (Table IV) confirm the activity of 15 enzymes out of 19 which were tested. Cystine arylamidase, β-galactosidase, α-mannosidase, and α-fucosidase were not detected. Esterase (C4) and esterase lipase (C8) were detected in all bacterial strains except H8.

Twelve strains exhibited α-glucosidase activity (M1, M4, H5, H6, H9-H13, S14, S16, S17). Nine strains exhibited naphtol-AS-BI-phosphohydrolase activity (M1-M3, H6, H8, H9, H11, H13, S16) and leucine arylamidase activity (M1, M3, M4, H9, H11-H13, S14, S16). Alkaline phosphatase was detected in seven strains (M3, M4, H8, H10, H12, S14, S16) and acid phosphatase – in six strains (M3, H8, H10-H13). Lipase C14 (S16), valine arylamidase (H11), trypsin (M3), α-chymotripsin (M3), α-galactosidase (H11), β-glucuronidase (H6), β-glucosidase (S16) and N-acetyl-β-glucosaminidase (S16) were detected only in single bacterial strains.

Molecular phylogenetic identification of strains revealed low diversity of cultivable bacteria, which belonged to four genera only (Fig. 1). *Microcella*, *Planococcus,* and *Halomonas* strains were isolated from the slightly saline samples, while *Bacillus* and *Halomonas* strains were isolated from the moderately and highly saline samples. In general, nine strains belonged to the genus *Bacillus*, six to *Halomonas*, while *Microcella* sp. and *Planococcus* sp. were represented by single isolates. The predominance of *Bacillus* species was the most significant in the highly saline samples.

Discussion

Alkaliphiles are a class of extremophiles which coexist with neutrophiles and inhabit extreme environments (Horikoshi, 1991). The number of alkaliphilic microorganisms in neutral soil samples ranges from 10^2 to 10^5 CFU g^{-1}, which corresponds to between 1/10 and 1/100 of the population of neutrophilic microorganisms (Horikoshi, 1991). In our study the ratio was higher, ranging from 1/2 to 1/7. The number of culturable aerobic bacteria in alkaline lime was approximately 10^5 CFU ml^{-1} in the alkaline medium. Our results are

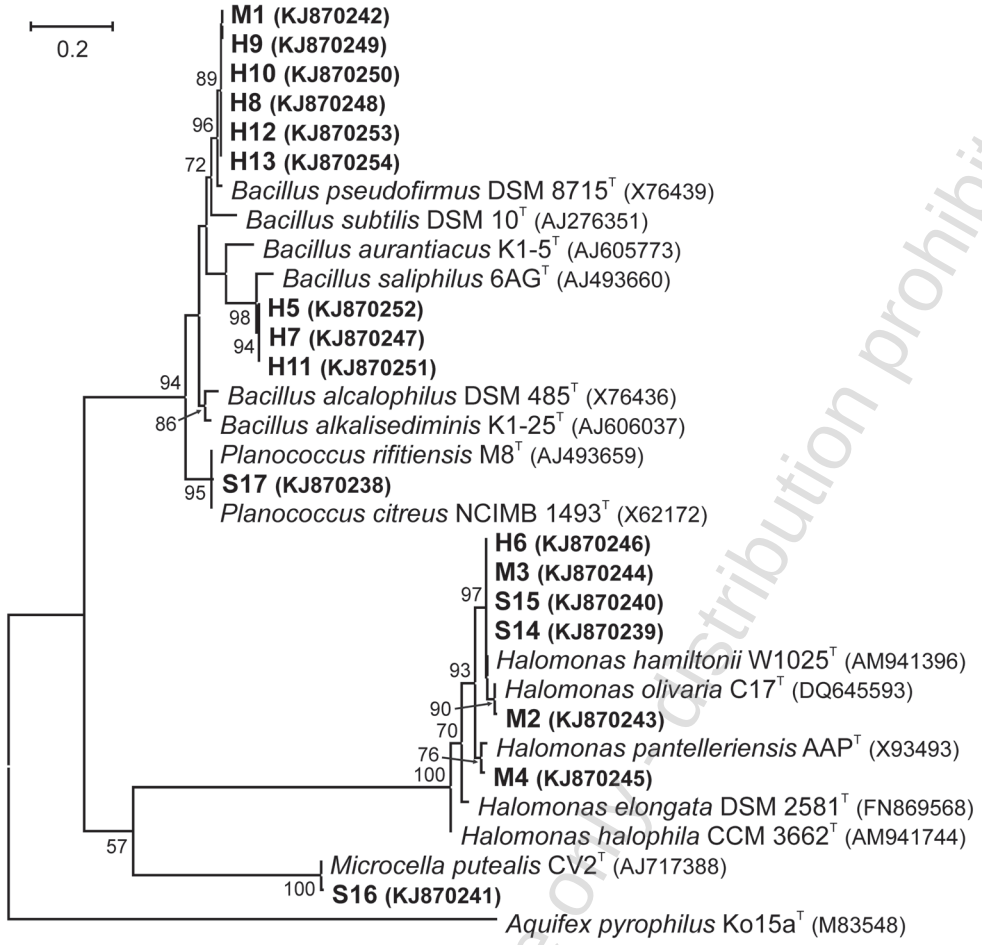

Fig. 1. Maximum likelihood phylogenetic tree based on the 16S rRNA gene including soda waste bacterial isolates and closely related type strains.

Explanations: The tree was reconstructed based on 781 nucleotide positions with the Tamura 3-parameter nucleotide substitution model. Bootstrap values lower than 50 were removed from the nodes. Sequences determined in this study appear in bold.

similar to those obtained by Ntougoias *et al.* (2006), who studied alkaline alpeorujo, a sludge-like by-product in olive oil extraction. However, the total number of heterotrophic bacteria in neutral medium was lower by two orders of magnitude in the alkaline lime. The difference may be caused by a considerably higher content of organic matter in alkaline alpeorujo (olives, olive leaves, and freshwater are a source of neutrophiles) compared to the nutrient-poor alkaline lime, consisting almost entirely of calcium carbonate.

The total number of bacteria in alkaline lime (approx. 10^7 cells g^{-1}) was higher than the total number of bacteria in carbonate rock cores collected from cave walls in the Bahamas (Schwabe *et al.*, 1997). On the other hand, it was lower compared to dolomites and soil, where it ranged from 10^8 to 10^9cells g^{-1} (Ellis *et al.*, 2003; Su *et al.*, 2004; Maron *et al.*, 2006; Stres, 2007; Sheibani *et al.*, 2013).

The present study is the first attempt to investigate the distribution of bacteria in alkaline distillery lime. The results of the physicochemical analyses indicate that lime is a very harsh environment due to high pH and low nutrient level. Depositing alkaline lime for over fifty years has promoted the selection of microorganisms capable of growing in a nutrient-poor, highly alkaline, and moderately saline environment. As a result, the majority of the bacterial strains isolated from the lime samples were facultative alkaliphiles and moderate halophiles.

Bacillus pseudofirmus, moderate halotolerant and obligate alkaliphile, first isolated from soil and animal manure (Nielsen *et al.*, 1995), was represented by six isolates (M1, H8, H9, H12, H13). These extremely halotolerant, facultatively alkaliphilic bacteria show marginal growth at pH 7 and optimum growth at the temperature of 20–30°C.

The closest relative of three isolates: H5, H7 and H11 was *Bacillus luteus*, first isolated from soil sample collected from Mandpam, Tamilnadu, India (Subhash *et al.*, 2014).

Planococcus rifietoensis, the closest relative of strain S17, is a halotolerant, alkaliphilic bacterium, first isolated from the algal mat formed at the sulfurous spring in Rifieto, Italy (Romano *et al.*, 1996).

Halomonas sp. are ubiquitous, moderately halophilic/halotolerant Gram-negative bacteria found in saline lakes, saline soils, marine environments, and solar salt extraction facilities. Several alkaliphilic species of *Halomonas* can live in soda lakes and alkaline soils (Máthé *et al*., 2014). *Halomonas hamiltonii*, the closest relative of strains M3, H6, S14, and S16, is a halophilic, facultative alkaliphilic rod-shaped bacterium, isolated from patients' blood and dialysis machines in a nursing home (Kim *et al*., 2010). *Halomonas olivaria*, the closest relative of bacterial strain M2 is a moderately halophilic, alkalitolerant bacterium, isolated from olive-processing effluents (Amouric *et al*., 2014). *Halomonas pantelleriensis,* the closest relative of strain M4, is a haloalkaliphilic rod-shaped bacterium with optimum growth at pH 9.0, isolated from hard sands of the lake Venere in the Pantelleria island, Italy (Romano *et al*., 2003).

Microbacteriaceae are widespread in natural and artificial environments such as soil, sewage, seawater and fresh water, plants, mushrooms, insects, and dairy products (Kageyama *et al*., 2007) although they have been rarely isolated from alkaline environments. They include *Microcella putealis*, the closest relative of strain S16, a Gram-positive alkaliphilic bacterium isolated from a non-saline, highly alkaline groundwater (Tiago *et al*., 2006).

The few available reports concerning microbial diversity of alkaline environments indicate the existence of many physiological groups (Duckworth *et al*., 1996; Jones *et al*., 1998; Horikoshi, 1999; Ntougias *et al*., 2006; Felföldi *et al*., 2009). The aerobic microbial population of these ecosystems contains organotrophic bacteria, including members of the *Bacillus* species, which are able to produce a wide range of enzymes used for recycling biopolymers: proteases, lipases, amylases, and cellulases. Strains capable of decomposing starch were the most numerous among the bacteria isolated from alkaline lime. Seventy percent of the isolated strains produced α-glucosidase, which breaks down starch and disaccharides to glucose. Bacterial amylases were extensively investigated because of their wide application in detergent, food, textile, and paper industries. Amylases with pH optimum of 9–12 have been identified in several alkaliphilic *Bacillus* spp. (Wang *et al*., 2006; Fujinami *et al*., 2010; Raval *et al*., 2014).

In addition to amylotylic strains, the investigated alkaline lime contained bacteria capable of hydrolyzing proteins. More than half of the bacterial strains showed leucine arylamidase activity and one of these strains synthesized trypsin and α-chymotrypsin. Alkaline proteases secreted by both neutrophilic and alkalophilic bacteria seem interesting because they constitute a major source of proteolytic enzymes produced commercially (Horikoshi, 1999; Fujinami *et al*., 2010). Alkalophilic proteases are widely applied in the detergent manufacturing (Wang *et al*., 2006), leather tanning (Vijay Kumar *et al*., 2011), food processing (Klomklao *et al*., 2012), and recovering silver from Xray films (Shankar *et al*., 2010). *Bacillus* species are main producers of many alkaline proteases, already isolated and described.

Bacteria which are able to synthesize lipase and decompose olive oil containing long chain fatty acids constituted the least numerous physiological group among the bacterial strains isolated from alkaline lime. Four isolates growing on agar plates with olive oil and Rhodamine B (indicator) hydrolyzed the substrate, releasing free fatty acids (producing an orange halo around the bacterial colonies). Surprisingly, the results of the APIZYM test revealed that only strain S16 showed (low) activity of lipase (C14). On the other hand all strains except one synthesized lipase/esterase (C8), which catalyze the hydrolysis of short chain fatty acids. Numerous applications of lipases include organic synthesis (Jiang *et al*., 2013), hydrolysis of fats and oils (Bourlieu *et al*., 2012), modification of fats (Gupta *et al*., 2003), flavor enhancement in food processing (Chávez *et al*., 2011), resolution of racemic mixtures, and chemical analyses (Sharma *et al*., 2011).

As has been demonstrated in several studies, some alklaliphilic *Bacillus* spp. produce cellulases which can be used for improving the efficiency of detergents (Ito, 1997). However, bacterial strains isolated from alkaline lime were unable to decompose carboxymethyl cellulose (CMC).

The comparison of taxonomic identification of the investigated microorganisms with their physiological and ecological properties and the activity of identified enzymes indicates that they were highly variable within the members of the same species (*e.g.* H5, H7 and H11 or H6, M3, S14 and S15). One the other hand, protein and lipid hydrolysis were detected only in strains that belonged to the genus *Bacillus* (and also in a *Microcella* strain).

The pilot data presented shows that a viable alkaliphile community is present in the highly alkaline, distillery lime, a by-product in Solvay' soda process. The species composition of the microbial population strongly depends on pH values and salt concentration. Moreover, alkaline lime is a poor in nutrients, oligotrophic environment. Autochthonous microorganisms can survive in these unfavorable conditions owing to their ability to form spores. Therefore the dominant genus among cultivable bacteria is *Bacillus*. The surface layer of the lime may be colonized by microorganisms transferred from the surrounding saline soils and Lake Pakoskie, situated 500 m West of the repository ponds.

The obtained results confirm that the isolates display various enzymatic activity; they can decompose starch, protein and fat. Past experiences at such

environments has shown that it is extremely likely that alkaline adapted and tolerant microbes may be useful for man. The results of this study will provide foundation for further detailed research on extremozymes secreted by the isolates.

Applied in this research culture-based method are naturally biased in their evaluation of microbial genetic diversity by selecting a small (< 1%), particular population of microorganisms present in a given environment. However the use of cultural enrichment techniques has its place for screening and isolating organisms for potential biotechnological application where industrial-scale growth in process systems, and hence amenability to growth in such systems, is required (Ritz, 2007).

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